A CAROTENE PRECURSOR: ITS PROPOSED STRUCTURE AND PLACE IN BIOSYNTHETIC SEQUENCE [1, 2]

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The isoprenoids, cholesterol and rubber, are known to arise from mevalonic (3,5-dihydroxy-3-methylvaleric) acid (5, 7). The same is true of carotenes (1, 6). Thus it has been found in this laboratory that mevalonic acid is rapidly incorporated into carotenes and related compounds in the tomato fruit (6). The hydrocarbon fraction of tomatoes previously incubated with 2-C^{14}-mevalonate contains radioactivity, however, not only in the known carotenes but also in a previously undescribed, colorless polyene which possesses absorption maxima at 208 mμ and 231 mμ. The high specific activity of this compound, which has been referred to as fraction II (6), suggests that it may be a precursor of carotenes. This paper reports further experiments pertaining to the identity of fraction II and to its role in the biosynthesis of carotenoids.

MATERIALS AND METHODS

SUBSTRATES: Samples of 2-C^{14}-mevalonic acid were obtained from Isotopes Specialties Co., Inc.[1] (Lot #A39111307, 0.92 mc/mM) and from Tracerlab, Inc.[5] (Lot 471-57-20, 1.1 mc/mM) as the N,N'-dibenzylethylene-diamine salt.

RADIOACTIVE ASSAY: C^{14}-containing samples were assayed for radioactivity with a Nuclear-Chicago Corp. Model D47 Micromil gas flow counter.[5] The small amounts of material used made correction for self-absorption unnecessary.

SUBSTRATE ADMINISTRATION: Fruits of commercial tomato varieties were supplied with substrate by injection into the locules by syringe or by vacuum infiltration through the stem scar.

CAROTENE EXTRACTION AND SEPARATION: The tomatoes, after incubation, were ground in methanol, and the carotene-containing hexane extract prepared by the method of Purcell et al (6), with the alteration that the xanthophylls were removed with 95 % methanol rather than upon a silica-methanol column. The hexane extract was chromatographed on 1.8 cm. by 25 cm. columns of magnesium oxide (Fisher Sea Sorb)[5] and Hyflo SuperCel (1 : 1, w : w). The various polyenes were eluted with progressively more polar solvent mixtures, beginning with hexane and ending with 2 % methanol : 10 % acetone : 88 % hexane. Carotenes were eluted from the column in the order of increasing unsaturation, the most highly unsaturated, lycopene, being discharged last. The separation of fractions Ia, Ib, IIa, and IIb from the crude phytoene mixture by chromatography on activated alumina has previously been described (6).

ULTRAVIOLET AND INFRARED SPECTRA: Ultraviolet spectra were obtained on hexane solutions with the Cary Model 11MS Recording spectrophotometer[5]. Infrared spectra were taken with the Perkin-Elmer 137 Infracord Spectrophotometer[5] using sodium chloride cells and chloroform as the solvent.

DEGRADATION PROCEDURES: Ozonolysis of fraction II was carried out by bubbling a stream of oxygen containing approximately 1 % ozone through the sample which was dissolved in 10 ml of chloroform at 0° C. The effluent gas was then led through a trap filled with an aqueous solution of potassium iodide and boric acid. When the sample no longer absorbed ozone, as indicated by the appearance of free iodine in the trap, 0.1 ml of acetic acid was added and ozonization continued for a further 3 minutes. The ozonides were next shaken with 0.2 ml of 30 % H_2O_2 for 15 minutes. This was followed by shaking with a further 0.15 ml of H_2O_2 for 15 additional minutes. Finally, 1.0 ml of H_2O_2 and 1.5 ml of water were added, and the mixture refluxed for 7 hours. Washed air was then bubbled through the mixture and into a solution of 2,4-dinitrophenylhydrazine reagent to trap any volatile carbonyl compounds that might be present. The remaining ozonolysis mixture was acidified with HCl and extracted with ether. Both the ether extract and the aqueous residue were examined by paper chromatography.

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[5] Mention of trade products and brands does not imply that they are endorsed or recommended by the U.S. Department of Agriculture or the California Institute of Technology over similar products and brands not mentioned.

TABLE I

POLYENES PRESENT IN TOMATOES AT DIFFERENT STAGES OF RIPENESS*

FRACTION	STAGE			
	MATURE GREEN	LIGHT PINK**	DARK PINK	TABLE RIPE
Fraction Ia	...	16.6	5.9	5.7
Fraction Ib			5.7	11.8
Fraction IIa	0.345	0.24	0.075	0.029
Fraction IIb	0.293		0.078	...
Phytoene	0	0.067	0.162	1.36
Phytofluene	0	0.005	0.135	0.54
Neo-β-carotene	0.0038***	0.005	0.0043	0.0018
β-Carotene	0.14	0.43	0.35	0.323
ζ-Carotene	0		0.061	0.027
γ-Carotene	0	0.018	0.094	0.025
Lycopene	0	0.085	1.34	6.89

* Quantities expressed in mg/100 g fresh weight.
** This stage is known commercially as the breaker stage.
*** May be mostly α-carotene.

RESULTS

KINETICS OF CAROTENE LABELING: The data previously reported have shown, as pointed out above, that when 2-C^{14}-mevalonic acid or 1-C^{14}-acetic acid are administered to ripening tomatoes, substantial amounts of radioactivity are found in a previously unreported compound, fraction IIa (6). Further experiments have been carried out to test the possibility that fraction IIa may be a carotene precursor. Thus table I concerns experiments in which 2-C^{14} mevalonic acid was supplied to tomato fruit at varying stages of fruit development. The data show that although many of the known carotenes increase from negligible amounts at the time of first color formation by the fruit to rather large amounts at full ripeness, fraction IIa decreases in quantity during this period. Immature green tomatoes contain even more fraction IIa, by a factor of three to four, than fruit at the beginning of color formation. The incorporation of 2-C^{14}-mevalonic acid or of 1-C^{14}-acetate into fraction IIa by such immature fruit is greater than that by mature fruit. Thus at the unripe stage, fraction IIa is being synthesized by the fruit at a rapid rate.

All attempts to bring about a direct conversion of radioactive fraction IIa to carotenes by injecting the former into ripening fruit have met with failure. This may very well be due to the almost complete insolubility of fraction II in aqueous media and to associated permeability difficulties. A different type of experiment has, therefore, been designed to test the fate of fraction IIa. The experiment is based upon the fact that the compound is rapidly synthesized in green fruit. It must first be noted that the incorporation of injected 2-C^{14}-mevalonic acid into non-saponifiable matter is essentially completed within 24 hours (table II). The labeled substrate which remains in the fruit is unavailable to metabolism, possibly due to its accumulation into vacuoles. Experiments in which fruits are injected a single time with mevalonate are therefore *pulse* experiments in which the transfer of label from initial product to successive metabolites of this product may be followed with time. Immature fruit were therefore fed 2-C^{14}-mevalonate and allowed to remain on the plant until color development had begun. Table III compares the distribution of activity among the carotene fractions of tomatoes injected with labeled mevalonate while immature and then incubated for various times either on or excised from the plant. Fruit incubated on the plant show somewhat less total incorporation of mevalonic acid into carotenes due to translocation of label to other parts of the plant.

The radioactivity of 2-C^{14}-mevalonate, after injection into the green fruit, is rapidly incorporated into fraction II of the crude phytoene fraction. Within 6 days after injection, however, the bulk of radioactivity has disappeared from this fraction and has appeared in a fraction which may be eluted from the magnesium oxide-SuperCel column immediately before β-carotene. This fraction remains highly labeled until the commencement of ripening. At this time it loses radioactivity which appears instead in the carotenes proper.

NATURE OF LABELED METABOLITES: Characterization studies have been carried out on the compounds isolated from the crude phytoene fraction of ripening

TABLE II

INCORPORATION OF 2-C^{14}-MEVALONATE INTO NON-SAPONIFIABLE FRACTION OF RIPENING TOMATOES INCUBATED FOR VARIOUS LENGTHS OF TIME

FRUIT WT	SUBSTRATE INJECTED	INCUBATION TIME	INCORPORATION	INCORPORATION/ 100 G FRESH WT
G	CPM	HR	CPM	%
177	1×10^6	4	52,000	2.9
250	1×10^6	24	159,000	6.4
290	1×10^6	48	165,400	5.7
200	1.8×10^6	170	183,000	5.1

tomatoes. Fractions Ia and Ib were grouped together for study because of their similarity in physical characteristics and because of the difficulty in securing a clean chromatographic separation of one from the other. Although it is present in large quantity in both immature and ripe tomatoes, fraction I does not become labeled in fruit injected with 2-C^{14}-mevalonic acid. The fact that 1-C^{14}-acetate is actively incorporated into fraction I indicates that this material may be a straight chain hydrocarbon derived from a fatty acid. In agreement with this view is the fact that ozonolysis of purified fraction I causes no detectable degradation. The molecule is, therefore completely saturated. Molecular weight, as determined by the Rast method (Mr. G. Swinehart) is 327, corresponding to a C_{23} or C_{24} compound. Carbon-hydrogen analysis (Dr. A. Elek) yielded the following duplicate results: C = 82.02 %, H = 12.96 %; C = 81.90 %, H = 13.05 %. The 5 % unaccounted for is sufficiently large to allow for an oxygen atom, but there is no indication from infrared spectra or from chromatographic behavior that an oxygen-containing group is present.

Much of the radioactivity of the phytofluene and neo-β-carotene fractions is due to the presence of an unidentified compound. This colorless substance has no absorption maxima above 208 mμ, melts in the range 140 to 150° C, partitions to the extent of 1 : 4 between 95 % methanol and hexane and is not precipitated by digitonin. These properties together with its infrared absorption spectrum suggest the compound may be a saturated xanthophyll.

Fractions IIa and IIb are not readily separated from one another on alumina. When either one was isolated and rechromatographed, small amounts of the other were obtained by rechromatography. This behavior suggested that the two may be isomers. Similar behavior was noted during the chromatographic purification on alumina of phytadiene as well as with farnesene.

Because of the apparently reversible conversion of IIa to IIb and because of the difficulty of obtaining large amounts of either material due to instability, the two compounds were not in general separated from one another for the further studies.

The ultraviolet spectrum of fraction II, which shows a characteristic maximum at 231 mμ (specific abs. coefficient = 55.4), indicates the presence of a pair of conjugated double bonds. The theoretical absorption maximum for a compound of this type is, according to Woodward's rule (9), at 227 mμ, but shifts of several mμ have been found by Woodward and by O'Connor and Goldblatt (4) for various substituted dienes. Non-conjugated systems do not exhibit appreciable absorption above 210 mμ, and trienes may be distinguished by their three maxima in the 260 mμ to 280 mμ region.

The infrared spectrum of fraction II furnishes evidence that the compound is of an isoprenoid nature. In the fingerprint region of 4,000 cm^{-1} to 1,000 cm^{-1} the peaks characteristic of conjugated diene terpenes are matched very closely in position and intensity by the maxima of the fraction II spectrum. It may be particularly noted that the infrared spectrum of fraction II exhibits a wide band at 1,600 cm^{-1} corresponding to that expected of a compound containing both conjugated and non-conjugated double bonds. A prominent peak at 800 to 840 cm^{-1} is consistent with the presence in the compound of a propylidine group. A further peak at 890 cm^{-1} corresponds to that expected of a compound containing a methylene group.

Molecular weight determination by the Signer method (Truesdail Labs.), yielded duplicate values of 297 and 284. The average weight of 290.5 is in reasonable agreement with that expected for a C_{20} isoprenoid compound (expected—272).

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN CAROTENE AND RELATED FRACTIONS OF TOMATO FRUITS INJECTED WITH 2-C^{14}-MEVALONIC ACID WHILE IMMATURE AND INCUBATED FOR PERIODS INDICATED

FRACTION	FRUIT INCUBATED 1 DAY HARVESTED GREEN	FRUIT INCUBATED 6 DAYS HARVESTED GREEN	FRUIT INCUBATED 12 DAYS HARVESTED DARK PINK	FRUIT INCUBATED 17 DAYS HARVESTED DARK PINK
Crude phytoene	50.5	2.2	2.8	4.3
Phytofluene)			42.2	7.3
)	10.6	68.7		
Neo β-carotene)			4.6	3.7
β-carotene	1.5	9.5	25.6	38.8
ζ-carotene *	6.8	...	21.2	20.2
Minor pigments +	...	...	2.0	0.9
γ-carotene	...	...	0.2	0.6
Lycopene	...	...	0	0.4

Results expressed as activity in each fraction as a per cent of the total activity in the non-saponifiable fraction.
* Contains radioactive sterols.
+ Probably a mixture of several pigments (8).
− No carotenes detected spectrophotometrically.

Samples of radioactive fraction II, containing principally fraction IIa but small amounts of fraction IIb, were degraded by ozonolysis. A radioactive volatile fragment was trapped in 2,4-dinitrophenylhydrazine reagent. The derivative was isolated and chromatographed on Whatman No. 3 paper, using the solvent systems: 5 % ether: 95 % hexane, and 80 % ethanol: 20 % hexane. The behavior of the compound in these solvents was found to be identical with that of authentic 2,4-dinitrophenylhydrazone of acetone. The recovery of the 2,4-dinitrophenylhydrazone of acetone was, however, low due no doubt to loss of the volatile degradation product during ozonolysis.

From the non-volatile ozonolysis residue relatively large amounts of labeled levulinic acid and labeled malonic acid were isolated by chromatographic procedures. These were identified by their chromatographic behavior on Whatman No. 1 paper in the five solvent systems: *n*-propanol 6-conc. ammonium hydroxide 4; ethanol 30-water 15-conc. ammonium hydroxide 5; ether 13-acetic acid 3-water 1; *n*-butanol 1-pyridine 1-water 1; and water saturated with *n*-butanol 95-formic acid 5. All attempts to determine the specific activities of the three labeled ozonolysis fragments of fraction II were unsuccessful due to the large inaccuracies involved in measurement of the small amounts of materials involved.

An isoprenoid structure which is derived from 2-C^{14}-mevalonic acid without randomization, should yield radioactive acetone, levulinic acid, and malonic acid upon ozonolysis and which would also be expected to possess other known properties of fraction II is proposed in figure 1. A comparison of the expected yields of radioactivity in the various ozonolysis fragments of the structure shown in figure 1 with those found in two separate degradation experiments are presented in table IV. These yields are low as yields from such degradation are often found to be.

TABLE IV

EXPECTED (ON BASIS OF STRUCTURE OF FIG 1) AND ACTUAL YIELDS OF RADIOACTIVITY IN OZONOLYSIS FRAGMENTS OF FRACTION II FORMED FROM 2-C^{14}-MEVALONIC ACID IN TOMATO FRUITS

	EXPECTED		FOUND	
	CPM	% TOTAL	CPM	% TOTAL
(a) SAMPLE OF FRACTION II CONTAINING 342,000 CPM				
Acetone	86,000	25	9,100	2.7
Levulinic acid	172,000	50	63,800	22.4
Malonic acid plus unknown	86,000	25	30,100	8.8
(b) SAMPLE OF FRACTION II CONTAINING 239,000 CPM				
Acetone	60,000	25	4,074	1.7
Levulinic acid	120,000	50	51,516	21.6
Malonic acid plus unknown	60,000	25	46,000	19.2

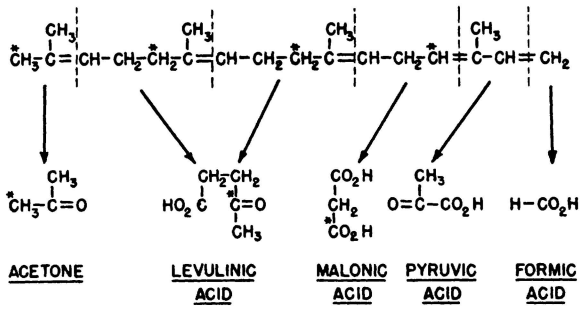

FIG. 1. Proposed structure of fraction II showing expected position of the label from 2-C^{14} mevalonate in fraction II and its ozonolysis products.

The structure of figure 1 should yield on degradation in addition to labeled acetone, levulinic acid and malonic acid, unlabeled pyruvic acid and an unlabeled oxidation product of the terminal methylene group. The terminal methylene group was determined by periodate-permanganate oxidation to formaldehyde (*2*) on a sample consisting entirely of fraction IIa. The yield of formaldehyde amounted to *79.2* % of that expected on the basis of the structure of figure 1. The presence of unlabeled pyruvic acid among the ozonolysis products has however not been rigorously authenticated.

DISCUSSION

Indirect evidence has been obtained for the participation of fraction II in carotene synthesis. The compound is formed rapidly during fruit development and appears to relinquish its radioactivity to carotenes during fruit ripening. Degradative studies as well as spectroscopic and chromatographic information have led to the formulation of a possible structure for fraction II. These studies have, however, been hampered by the difficulty of accumulating large amounts of fraction II, which is found in tomato fruit to the extent of less than one to several micrograms per gm of fruit.

Lynen et al (*3*) have reported the conversion of mevalonic acid to Δ^3-isopentenol pyrophosphate units, which condense to form farnesyl pyrophosphate and subsequently, squalene. An analogous series of reactions may be suggested for the biosynthesis of the carotenes. In this case, four isopentenyl residues may be visualized as condensing to yield the 20 carbon analog of farnesyl pyrophosphate. According to this suggested pathway, fraction II would be synthesized from mevalonate via Δ^3-isopentenol pyrophosphate. One molecule of fraction II would condense upon the pyrophosphate form of a similar C_{20} intermediate, forming a C_{40} skeleton which would at the time of fruit ripening be dehydrogenated to yield one or another of the carotenes proper. The primary C_{40} compound has, however, not yet been discovered. This may be because it is further transformed within the fruit as rapidly as it is formed.

Summary

A substance has been isolated from tomatoes which appears to be an intermediate in the pathway from mevalonic acid to the carotenes. The results of infrared spectroscopy and degradative studies indicate a probable structure of 3, 7, 11, 15 tetramethyl-hexadeca-1, 3, 6, 10, 14 pentaene.

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